Nuclear Hormone Receptor Drug Screens

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CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuing application under 35USC120 of US Application No. Serial No. 08/975,614, filed November 21, 1997 by the same inventors.

INTRODUCTION

Field of the Invention

The field of this invention is screens for drugs effecting nuclear hormone receptor function.

Background

Nuclear hormone receptors comprise a large, well-defined family of ligand-activated transcription factors which modify the expression of target genes by binding to specific cis-acting sequences (Laudet et al., 1992, EMBO J, Vol, 1003-1013; Lopes da Silva et al., 1995, TINS 18, 542-548; Mangelsdorf et al., 1995, Cell 83, 835-839; Mangelsdorf et al., 1995, Cell 83, 841-850). Family members include both orphan receptors and receptors for a wide variety of clinically significant ligands including steroids, vitamin D, thyroid hormones, retinoic acid, etc. Ligand binding is believed to induce a conformational change in the receptors and promote their association with transcriptional coactivators, which are a diverse group of large nuclear proteins (Glass et al., 1997, Curr Opn Cell Biol 9, 222-232), which may share a signature sequence motif (Heery et al., 1997, Nature 733-736). The resulting complex then binds high affinity sites in chromatin and modulates gene transcription.

The classic approach to identifying agonists or antagonists of nuclear hormone receptors is the ligand displacement assay, where the displacement of radiolabeled ligand by candidate agents is detected. An alternative approach is a cell-based transcription assay for expression of a reporter of nuclear hormone receptor activation (e.g. Evans et al. (1991) US Patent No.5,071,773). More recently, a gel-based coactivator dependent receptor ligand assay (Krey et al., 1997, Mol Endocrinol 11, 779-791) has been used to identify ligands of

peroxisome proliferator-activated receptors (PPARs), which are nuclear hormone receptors activated by a variety of compounds including hypolipidemic drugs. Unfortunately, these various assays suffer from a number of limitations including a required known ligand and time, labor and resource intensive cell-based and gel-based methods, respectively.

SUMMARY OF THE INVENTION

The invention provides methods and compositions for efficient screening of modulators of nuclear hormone receptor function, without the use of cell- or gel-based steps. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for bioactive compounds.

In one embodiment, the invention provides in vitro methods comprising the steps of (a) forming a mixture comprising a nuclear hormone receptor, a peptide sensor and a candidate agent, but not a natural coactivator protein of the receptor, wherein the sensor provides direct, in vitro binding to the receptor under assay conditions; (b) measuring an agent-biased binding of the sensor to the receptor; and (c) comparing the agent-biased binding with a corresponding unbiased binding of the sensor to the receptor, wherein a difference between the biased and unbiased bindings indicates that the agent modulates a receptor function. In particular embodiments, the sensor comprises an amphipathic alpha helix nuclear hormone interacting domain comprising a nuclear hormone transcriptional coactivator motif sequence. To ensure specificity and optimize binding, the sensor is generally present at sub-micromolar concentration and the binding reaction occurs in solution. In a preferred embodiment, the sensor comprises a fluorescent label and the measuring step comprises detecting fluorescence polarization of the label.

The invention also provides reagents such as labeled sensor peptides and reaction mixtures consisting essentially of nuclear hormone receptor, a peptide and a candidate agent, wherein the peptide provides direct, in vitro ligand-dependent binding to the receptor, especially in which the binding is enhanced in the presence of the agent.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. LXR activation dose response with oxysterol ligands in fluorescent polarization assay with TUK-1391 sensor.

Figure 2. Dose response showing 24-ketocholesterol ligand (2 μM) increases LXR receptor affinity for labeled peptide sensor in fluorescent polarization assay.

Figure 3. Dose response showing 9-cis-retinoic acid ligand (1 μM) increases RXR receptor affinity for labeled peptide sensor in fluorescent polarization assay.

Figure 4. Fluorescent polarization NRH agonist assay validation for LXR/24-ketocholesterol ligand (2 μM), PPARγ/BRL49653 (1 μM) and RXR/9-cis-retinoic acid ligand (1 μM) with TUK-1391 sensor.

DETAILED DESCRIPTION OF THE INVENTION

The methods generally employ a mixture comprising three components: a nuclear hormone receptor, a peptide sensor and a candidate agent, in amounts effective to measure the targeted interactions. Many natural nuclear hormone receptors are modular proteins with discrete functional domains, including a ligand binding domain; see Laudet et al.; Lopes da Silva et al.; Mangelsdorf et al.; supra. The subject receptors encompass such full-length receptors as well as portions of the receptors sufficient to provide differential sensor binding in the presence and absence of a corresponding receptor ligand, agonist and/or antagonist. Such portions generally comprise at least the ligand binding domain of the receptor. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, *et al.* Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, *et al.*, Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art. Exemplary nuclear hormone receptors and corresponding target therapeutic application are listed in Table 1.

Table 1. Exemplary nuclear hormone receptors, form (M = monomeric, D = heterodimeric, H = homodimeric), tissue expression and target therapeutic application.

Receptor	Form	Tissue Expression	Target Therapeutic Application
NURR1	M/D	Dopaminergic Neurons	Parkinson's Disease
RZRβ	M	Brain (Pituitary), Muscle	Sleep Disorders
RORα	M	Cerebellum, Purkinje Cells	Arthritis, Cerebellar Ataxia

NOR-1	M	Brain, Muscle, Heart, Adrenal, Thymus	CNS Disorders, Cancer
Rev-ErbAβ	H	Brain, Muscle, Spleen	CNS Disorders
Tlx	H	Embryonic and Adult Brain	CNS Disorders
NGFI-Bβ	M/D	Brain	CNS Disorders
HZF-2α	H	Hippocampus	CNS Disorders
COUP-TFα	H	Brain	CNS Disorders
COUP-TFβ	H	Brain	CNS Disorders
COUP-TFγ	H	Brain	CNS Disorders
Nur77	M/D	Brain, Thymus, Adrenals	CNS Disorders
LXRα	D	Liver, Kidney, Spleen, Adrenals	Hypercholesterolemia
COR	M	Liver, Pancreas	Hypercholesterolemia
Rev-ErbAα	H	Muscle, Brain (Ubiquitous)	Obesity
HNF4α	H	Liver, Kidney, Intestine	Diabetes
TOR	M	Thymus, T Cells, Lymphoma	Immune Disorders
MB67α	D	Liver	Metabolic Disorders
SHP	D	Liver, Heart, Pancreas	Metabolic Disorders
FXR	D	Liver, Kidney	Metabolic Disorders
SF-1	M	Gonads, Pituitary	Metabolic Disorders
LXRβ	D	Kidney (Ubiquitous)	Metabolic Disorders
GCNF	M/H	Testes, Ovary	Infertility
TR2-11α,β	H	Testes	Infertility, Contraception
TR4	H	Testes	Infertility, Contraception
ERRα,β	M	Placenta	Infertility
DAX-1	M	Testes, Adrenals, Ovary, Liver	Adrenal Hypoplasia, Hypogonadism

The mixture also includes a peptide sensor which provides direct, in vitro, significant assay detectable binding to the receptor under assay conditions. Accordingly, the sensor obviates the need to include a natural coactivator protein of the receptor in the mixture. The sensor comprises a receptor binding sequence, generally $L_1X_1X_2L_2L_3$, wherein L_1-L_3 are independently selected from hydrophobic amino acids, preferably leucine or isoleucine, more preferably leucine; and X_1-X_2 are independently selected from any amino acid, preferably any natural amino acid. The sensor region comprising this sequence generally forms an

amphipathic alpha helix. Such sequences may be natural coactivator protein motif sequences, derived from coactivator motif sequences or consensus sequences thereof, e.g. by step-wise mutational analysis, and/or from screens of purely or partly synthetic sequences, e.g. randomizing residues and selecting for receptor binding. The sensors are of length and sequence sufficient to effect the requisite specific binding, generally 50 or fewer, preferably 24 or fewer, more preferably 12 or fewer residues in length. Accordingly, panels of predetermined or randomized candidate sensors are readily screened for receptor binding. For example, in the high-throughput fluorescent polarization assay (below), candidate sensors demonstrating specific binding are conveniently identified by enhanced fluorescent polarization, generally an increase of at least about 5, preferably at least about 10, more preferably at least about 20 millipolarization units under optimized binding assay conditions.

In a particular embodiment, the sensors demonstrate ligand, agonist and/or ligand dependent binding i.e. the sensor differentially binds the receptor in the presence and absence of such ligand/agonist/antagonist, generally differential binding of at least 10/10%, preferably at least 100/50%, more preferably at least 1,000/90%, respectively. Accordingly, panels of predetermined or randomized candidate sensors are readily screened for differential binding, as exemplified in Figures 2 and 3 for two exemplary receptor/ligand pairs. Analogously, differential binding is conveniently demonstrated using known agonists or antagonists of targeted receptors. For orphan receptors, it is often convenient to prescreen known ligands for pseudo-ligands or surrogates which selectively bind the ligand binding domain. Alternatively, agonists and/or antagonists may be identified by screening predetermined or randomized candidate labeled peptides for sensors which demonstrate assay detectable receptor binding, and then screening for agents which increase/decrease the binding of the identified sensor to the receptor, i.e. agonists/antagonists, respectively.

Exemplary sensors and binding data are shown in Table 2.

Table 2. Sensors Activity: Fluorescent Polarization Assay

Sensor	Label	Sequence	LXR	PPARγ	RXR
SRC-1 632-640					
TUK-1384	F-	KLVQLLTTT (SEQ ID NO:1)	I	O	O
TUK-1386	F-G-	KLVQLLTTT	I	O	O
TUK-1385	R-	KLVQLLTTT	II+	I	II+
TUK-1387	R-G-	KLVQLLTTT	+	O	II

SRC-1 689-696						
	TUK-1370	F-	ILHRLLQE (SEQ ID NO:2)	II	O	II
	TUK-1371	R-	ILHRLLQE	IV	I+	IV
	TUK-1373	R-G-	ILHRLLQE	II	O	II+
SRC-1 748-755						
	TUK-1390	F-	LLRYLLDK (SEQ ID NO:3)	IV	II	II+
	TUK-1392	F-G-	LLRYLLDK	II+	I	I
	TUK-1391	R-	LLRYLLDK	IV	II+	IV
	TUK-1393	R-G-	LLRYLLDK	III+	I	II+
SRC-1 748-754						
	TUK-1453	R-	LLRYLLD (SEQ ID NO:4)	III	I	I+
SRC-1 749-754						
	TUK-1455	R-	LRYLLD (SEQ ID NO:5)	IV	I+	I
SRC-1 748-753						
	TUK-1457	R-	LLRYLL (SEQ ID NO:6)	II	+	I
SRC-1 749-753						
	TUK-1459	R-	LRYLL (SEQ ID NO:7)	III	I	O
SRC-1 748-756						
	TUK-1472	R-	LLRYLLDKD (SEQ ID NO:8)	IV	II	IV
SRC-1 747-756						
	TUK-1473	R-	QLLRYLLDKD (SEQ ID NO:9)	IV	I+	I+
SRC-1 746-756						
	TUK-1474	R-	HQLLRYLLDKD (SEQ ID NO:10)	IV	O	I
SRC-1 1427-1440						
	TUK-1395	F-	PQAQQKSLLQQLLT (SEQ ID NO:11)	O	O	O
	TUK-1397	F-G-	PQAQQKSLLQQLLT	O	O	O
	TUK-1398	R-G-	PQAQQKSLLQQLLT	O	O	O
SRC-1 1434-1441						
	TUK-1380	F-	LLQQLLTE (SEQ ID NO:12)	II+	O	O
	TUK-1382	F-G-	LLQQLLTE	II+	O	O
	TUK-1381	R-	LLQQLLTE	IV	I+	I+
	TUK-1383	R-G-	LLQQLLTE	II	O	O

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TUK-1374	F-	VTLLQLLLG (SEQ ID NO:13)	IV	I	O
TUK-1376	F-G-	VTLLQLLLG	II+	+	O
TUK-1375(1433)R-		VTLLQLLLG	IV	I	O
TUK-1377	R-G-	VTLLQLLLG	II	I	+

Synthetic sequence peptides

TUK-1560	R-	ILRKLLQE (SEQ ID NO:14)	IV	II	IV
TUK-1559	R-	ILKRLLQE (SEQ ID NO:15)	IV	0	IV
TUK-1558	R-	ILRRLLQE (SEQ ID NO:16)	III	0	IV
TUK-1557	R-	ILKKLLQE (SEQ ID NO:17)	III+	0	IV

The sensor also comprises a detectable label. A wide variety of labels may be used including labels providing for direct detection such as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc. In a particular embodiment, the label is differentially detectable according to receptor binding, obviating the need for any bound versus unbound separation step. In a more particular embodiment, the label is a fluorescent label which provides differential fluorescence polarization depending on receptor binding. Exemplary such labels include rhodamine and fluorescein, which may be coupled directly or indirectly though a linker, e.g. an amino acid linker. Suitable labels and methods for peptide conjugation/incorporation (e.g. during solid phase peptide synthesis) are well known in the art. The sensor is generally present at a concentration of less than about 1 μM, preferably less than about 100 nM, more preferably less than about 10 nM and most preferably less than about 1 nM.

The assay mixture also comprises a candidate agent. Suitable candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. In a particular embodiment, the assay mixture also comprises a known ligand of the receptor. This embodiment is particularly suitable for screening for antagonists of the receptor. A variety of other reagents may also be

included in the mixture. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, etc. may be used.

The mixture is incubated under conditions whereby, but for the presence of the candidate agent, the sensor binds the receptor with a reference binding affinity. In a particular embodiment, all the components of the mixture, including the receptor, peptide and agent, are in solution. The mixture components can be added in any order that provides for the requisite bindings and incubations may be performed at any temperature which facilitates optimal binding. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening. After incubation, the agent-biased binding between the sensor and receptor is detected according to the nature of the label, as described above. A difference in the binding in the presence and absence of the agent indicates that the agent modulates a receptor binding function. A difference, as used herein, is statistically significant and preferably represents at least a 50%, more preferably at least a 90% difference.

A wide variety of methods may be used to measure binding between the sensor and the receptor, depending on the nature of the sensor and nuclear hormone receptor, whether the assay is performed in solution or partly or fully in solid phase, whether luminescent, radioactive or fluorescent emissions are used, whether the sensor or the receptor of immobilized receptor-sensor complexes is detected, etc. In solid phase embodiments, the measuring step may involve, inter alia, immobilizing the receptor through the sensor or the sensor through the receptor. For example, the sensor is considered to comprise a label, such as a tag or the amino acids of the sensor itself may provide an epitope label. Hence, the receptor may be immobilized through the sensor and the sensor immobilized through the label. The label may be directly coupled to the solid phase by conventional covalent and/or noncovalent linkages, or be coupled indirectly through one or more second receptors, specific for the label, such as sensor epitope specific antibodies, avidin (where the label is biotin), etc. Where the sensor is so immobilized, the measuring step generally comprises detecting the immobilized nuclear hormone receptor, such as with a receptor-specific antibody, as in a conventional ELISA format. Preferred ELISA format assays employ a chemiluminescent or time-resolved fluorescent substrate for convenient readout, especially in high throughput applications.

In alternative solid phase embodiments, the sensor and receptor are reversed, i.e. the sensor is immobilized through the nuclear hormone receptor. Similarly, the receptor may be

directly immobilized, or coupled through one or more receptor-specific receptors and the immobilized sensor may then be detected directly or indirectly, e.g. in an ELISA-type format.

The solid phase assays are particularly useful when a plurality of different sequence sensors are sought to be screened simultaneously or in parallel, in panels or mixtures. For example, with orphan receptors, weakly binding peptides are identified in phage display binding assays. Mixtures of weakly binding peptides are used to screen libraries for compounds which modulate peptide binding, and positive mixtures are then separated and assayed by individual peptides. Such active peptides are then directly labeled, e.g. with a fluorescent moiety, for use, e.g. in extremely high throughput solution phase assays, such as the fluorescent polarization assay described below. Panels of such peptides may also be used to screen candidate modulators of known receptors, providing a series of binding effects which constitute a virtual fingerprint of the activity/specificity of a given modulator. In addition, such peptides may be used to identify preferred controls for solution phase assays which require a directly detectable sensor peptide.

The invention also provides reagents for use in the subject methods. For example, the invention provides sensors consisting of, or consisting essentially of, a peptide comprising the sequence $L_1X_1X_2L_2L_3$ covalently coupled to a detectable label, wherein L_1-L_3 are independently selected from hydrophobic amino acids and X_1-X_2 are independently selected from any amino acid and wherein the peptide provides direct, in vitro ligand-dependent binding to a nuclear hormone receptor. In a particular embodiment, the label is a fluorescent label coupled to the N-terminus of the peptide and the peptide is 24, preferably 18, more preferably 12, most preferably 8 or fewer residues in length. The invention also provides reagent mixtures, such as a mixture consisting essentially of nuclear hormone receptor, a peptide and a candidate agent, wherein the peptide provides direct, in vitro ligand-dependent binding to the receptor, preferably wherein the binding is enhanced in the presence of the agent.

The following example is offered by way of illustration and not by way of limitation.

EXAMPLES

I. <u>High-Throughput In Vitro Fluorescence Polarization Assay</u>

Reagents:

Sensor: Rhodamine-labeled $L_1X_1X_2L_2L_3$ peptide (final conc. = 1 - 5 nM)

Receptor: Glutathione-S-transferase/nuclear hormone receptor ligand binding domain fusion protein (final conc. = 100 - 200 nM)

Buffer: 10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6

Protocol:

1. Add 90 microliters of peptide/NHR mixture to each well of a 96-well microtiter plate.
2. Add 10 microliters of test compound per well.
3. Shake 5 min and within 5 minutes determine amount of fluorescence polarization by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc).

II. Conformational Sensor - ELISA Format Assay

Buffer and Solution Preparation:

1. 10X Assay Buffer:
   100mL of 1M Hepes
   300mL of 5M NaCl
   20mL of 1M MgCl
   Add MQ H2O to 1L
2. Master Mix of Ligand / peptide / protein
   Protein: final conc = 100 nM
   Biotin - peptide (MW: 1366.7387g/mole): final conc = 1 uM
   Add Assay Buffer and H2O to bring to final volume: final buffer conc = 1X
3. Antibody Mix:
   anti-GST, rabbit (final conc. = 1:10,000)
   anti-rabbit-HRP (final conc. = 1:10,000)
   Add T-TBS to bring to final volume: final buffer conc = 1X

Procedure:

1. Make 50 mL of Master Mix (see 2 above) of appropriate peptide / protein combinations (use 50 mL polypropylene tubes). Incubate for 1 hr at RT
2. Add 95 uL of Master Mix to each well of a 96-well plate**

** Reacti-Bind Streptavidin-Coated, White Polystyrene Plates (#15118B), which have been blocked by Super-Blocking Reagent from Pierce.

3. Transfer 5 uL of each test compound (stock = 60 uM) to each well of the plate
4. Incubate plate for 1hr at RT
5. While incubating, make rabbit anti-GST antibody and anti-rabbit-HRP Antibody Mix (see 3 above). Incubate on ice for 1 hr.
6. Wash plates 3X with H2O thoroughly
7. Add 100 uL of Antibody Mix into each well of the plate
8. Incubate for 1 hr at RT
9. Wash 3X with H2O
10. Dilute Supersignal substrate (mixed Luminol and peroxide) in 1:2 H2O and then add 100 uL into each well
11. Shake 3-5 min. Read chemiluminescence.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

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